STOMATAL REGULATION IN TRANSGENIC HYBRID ASPEN (*Populus tremula* L. × *P. tremuloides* Michx) EXPRESSING ARABIDOPSIS *SLAC1*

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Tiivistelmä — Referat — Abstract

Stomata are microscopic pores; surrounded by a pair of guard cells; they play a crucial role in minimizing the trade-off between conservation of water and photosynthetic efficiency. SLAC1, a stomatal anion channel protein mediates stomatal closure in response to elevated CO_2 concentration. Genetic evidence suggested that *Populus* SLAC1 might have lost its function. In this study hybrid aspen (*P. tremula* L. × *P. tremuloides* Michx.*)* clone 51 was transformed by introducing *Arabidopsis thaliana SLAC1* gene regulated by either *SLAC1* or *GC1* promoter. The aim was to find out guard cell specific promoter and to select transgenic lines that showed rapid stomatal closure in response to elevated CO_2 concentration. Histochemical GUS assay suggested that the *SLAC1* promoter is more guard cell specific than *GC1* promoter. The gas exchange experiment showed an overall decrease in stomatal conductance in transgenic lines with the increasing CO_2 concentration compared to wild type plants. However, it was difficult to select the strongest transgenic lines, as all the replicates of independent lines did not show clear response of stomatal closure; in response to elevated CO_2 concentration.

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Stomata, Hybrid Aspen, SLAC1, Gas Exchange

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LIST OF ABBREVIATIONS

ABA	Abscisic Acid
ANOVA	Analysis of Variance
Ca^{2+}	Calcium Ion
cDNA	Complementary DNA
CO_2	Carbon diOxide
DNA	Deoxyribonuleic Acid
EYFP	Enhanced Yellow Fluorescent Protein
GC1	Guard Cell 1
GUS	β-glucuronides
H_2O_2	Hydrogen per Oxide
HA	Human Influenza Hemagglutinin
NO	Nitric Oxide
OST1	OPEN STOMATA 1
PCR	Polymerase Chain Reaction
qPCR	Quantitative Real-Time PCR
RNA	Ribonuleic Acid
R-type	Rapid-type
SD	Standard Deviation
SE	Standard Error
SLAC1	SLOW ANION CHANNEL-ASSOCIATED 1
S-type	Slow-type

1 INTRODUCTION

Stomata are microscopic pores, found in the epidermis of plant leaves and stems that plays most important role in plant gas exchange. Stomatal pores are surrounded by a pair of kidney shaped guard cells which functions in opening and closing of stomata. Through opening and closing, stomata provide gates for exchange of water vapour and carbon dioxide (CO_2) between plants and the atmosphere, and thus maintain the global water and carbon cycle (Blatt 2000). In addition, adequate stomatal regulation limits the entry of pathogens (Melotto et al. 2006) and air pollutant such as ozone (O_3), which affects crop yields and natural vegetation (Vahisalu et al. 2008, Brosché et al. 2010). Stomatal opening and closing is influenced by different environmental factors such as light intensity and quality, soil water content, air humidity, CO_2 concentration and in response to air pollutants. With the changes of these one or more external factors, plants produce different hormones and signaling molecules to guide different physiological aspects including regulation of stomatal aperture. Turgor pressure of guard cells regulate the aperture of stomatal pore (Geiger et al. 2011, Negi et al. 2014). Stomata opens with an increment of turgor pressure in guard cells and closing of stomata occurs as a result of the decline of turgor pressure in guard cells (Hetherington and Woodward 2003).

The role of ion channel in plants is vital as mature guard cells are lacking plasmodesmata. Therefore the influxes and effluxes of osmotically active ions and metabolites occur via ion channels and ion transporters present in guard cell membrane (Pandey et al. 2007, Vahisalu et al. 2008). Anion channels play central role in signal transduction, nutrient transport and regulation of cell turgor (Barbier-Brygoo et al. 2000) and so far their functions have been studied extensively in the guard cells of stomata, using different combination of pharmacological, electrophysiological and genetic tools (Diatloff et al. 2010). Depending on the diverse mechanism, different types of ion channels have been reported. However, studies on guard cells mostly emphasized and characterized two types of voltage dependent anion channels; Rapid-activating (R-type) and Slow-activating (S-type) anion channels (Schroeder and Hagiwara 1989, Hedrich et al. 1990, Schroeder and Keller 1992), which have been reported in different

plant species such as *Vicia faba*, *Nicotiana* species and *Arabidopsis thalina* (De Angeli et al. 2007).

A gene named *SLOW ANION CHANNEL-ASSOCIATED 1* (*SLAC1*) was isolated from *Arabidopsis thaliana* and has been reported to be responsible for encoding the guard cell plasma membrane S-type anion channel (Vahisalu et al. 2008, Negi et al. 2008). The expression of *SLAC1* is highly specific to guard cells and the protein plays crucial role in stomatal closure, as many studies have indicated that plants lacking functional *SLAC1* exhibit impaired stomatal closure in response to different environmental stimuli such as CO_2, light, O_3 and humidity, and endogenous stimuli such as abscisic acid (ABA), calcium ion (Ca^{2+}), hydrogen per oxide (H_2O_2) and nitric oxide (NO) (Vahisalu et al. 2008, Negi et al. 2008, Saji et al. 2008, Vahisalu et al. 2010). Therefore, a *SLAC1* deficient plant would be an ideal and useful model to study the stomatal regulation under varying environmental conditions.

So far the mechanism of *SLAC1* regulation have been studied extensively at molecular level in Arabidopsis (Vahisalu et al. 2008, Vahisalu et al. 2010). However, preliminary results suggest that the *SLAC1* gene present in *Populus* species may have lost the functionally important regulatory and structural features and thus have altered the ability of rapid stomatal regulation (Sanna Ehonen, University of Helsinki, personal communication). This speculation is supported by the experiment done by Aasamaa and Sõber, (2011), where they found that in response to increasing CO_2 concentration the stomatal sensitivity for aspen (*P tremula* L.) was lower than in other species used in the experiment. Therefore, a detailed knowledge of stomatal regulation of *Populus* species and hybrids that are of considerable commercial importance at temperate region, are of utmost importance.

In this study transgenic hybrid aspen (*P. tremula* L. × *P. tremuloides* Michx) was used. Hybrid aspen clone 51 was transformed by introducing *Arabidopsis thaliana SLAC1* gene through *Agrobacterium* transformation. Different transgenic lines were produced for different purposes of this study. Transgenic lines containing *promoter::GUS* construct, were used to study and select a promoter that is highly specific to guard cells, and transgenic lines containing *promoter::AtSLAC1-tag*,

were used to find the most strong transgenic lines by studying how the introduced Arabidopsis *SLAC1* gene affects rapid stomatal closure in response to elevated CO_2 and growth in *Populus,* since *Populus* own *SLAC1* is most likely nonfunctional.

Transgenic lines containing *SLAC1* gene were all made with two different guard cells-specific promoters; *pSLAC1* and *GUARD CELL* 1 (*pGC1*), (Yang et al. 2008) and with two different tags; Human influenza hemagglutinin (HA) and Enhanced Yellow Fluorescent Protein (EYFP). The objectives of using these promoters were to study a guard cell-specific promoters in *Populus* and the specificity of these promoters in *Populus* (Sanna Ehonen, University of Helsinki, personal communication). The purpose of adding tags, either; HA or EYFP, were to observe the protein expression in the plant, with the help of western blot technique (HA and EYFP-tag) or by confocal microscopy (EYFP-tag). Two different tags were used as sometimes a protein tag may interfere the function of the protein. HA-tag, being smaller than EYFP-tag, is less likely to interfere protein function. However, with EYFP-tag, the tagged protein can be visualized under confocal microscopy to check that it is correctly localized to the guard cells membrane. Further studies will be conducted to select the better tag.

Different types of experiment were conducted in this study. The histochemical β-glucuronides (GUS) assay were performed to select guard cell-specific promoter. The relative expression of the transgene was investigated by quantitative real-time PCR (qPCR) method. The stomatal conductance of different transgenic lines and wild type plants were measured in response to increasing CO_2 concentration. The result of histochemical GUS assay showed that, *SLAC1* promoter is more guard cell-specific compared to the *GC1* promoter. The gas exchange measurement of the transgenic lines and wild types suggested that there was an overall decline of stomatal conductance in transgenic lines in response to elevated CO_2 concentration compared to wild type. However, it was very difficult to identify and select the strongest transgenic lines that showed a clear response to elevated CO_2 concentration, as all the biological replicates for an independent transgenic lines did not respond at the same way.

2 LITERATURE REVIEW

2.1.1 Biology of hybrid aspen

Both *Populus tremula* L. (common aspen, European or Eurasian aspen) and *P. tremuloides* Michx. (quaking or trembling aspen), parental species of hybrid aspen, are members of Salicaceae family, belonging to the genus *Populus* (which is divided in to six sections) and section *Populus* (Tullus et al. 2012). Both of the parental species have wide natural distribution ranges, where *P. tremula* is considered as one of the most largely distributed trees worldwide (Worrell 1995) and is the only endemic *Populus* species in Finland. On the other hand, *P. tremuloides* is the most widely distributed tree species that is native to North America (Tullus et al. 2012) Studies have shown that both of these species are genetically close (Cervera et al. 2005) and they can also be regarded as single species with circumboreal distribution (MacKenzie 2010). Although triploid and tetraploid individuals are found, aspens are commonly diploid (2n = 38). They are dioecious, medium sized trees and can propagate through seed or root suckering (Worrell 1995). Both of these species are economically important and their genetically variable nature with different geographic varieties and forms provide diverse material for breeding and selection (Li 1995).

Hybrid aspen, which has been made by artificial cross between *P. tremula* and *P. tremuloides,* was first reported in Germany at the beginning of the 1920s (Tullus et al. 2012) and in Finland it was first produced at the Ruotsinkylä field station of the Finnish Forest research institute in year 1950 (Yu et al. 2001). Hybrid aspen grows faster and shows higher biomass production compare to its parental species and it is confirmed in different experimental and commercial plantations in Scandinavia (Yu 2001) Central Europe (Tiefenbacher 1991, Liesebach et al. 1999) and North America (Li et al. 1993).

In northern regions, for example in Finland, hybrid aspen grows faster compare to local aspen due to longer growing season (Yu et al. 2001). Some other physiological traits related to hybrid vigor of hybrid aspen are larger stomatal guard cell with a lower stomatal density compared to *P. tremula* (Yu 2001) and higher net photosynthetic rate (Tullus et al. 2012). There is large variation in

growth, phenology, physiology and phytochemistry between different hybrid aspen clones (Yu et al. 2001, Rytter and Stener 2003, Yu and Pulkkinen 2003) providing greater possibilities in tree breeding (Stener and Karlsson 2004).

2.1.2 Ecological and economic importance

European aspen is ecologically important species in maintaining biodiversity, as several other species including animals, mosses lichens, and fungi depend upon it (Kouki et al. 2004). More specifically, in Finland, European aspen is one of the most important host for critically endangered species (Tikkanen et al. 2006). The higher calcium content of aspen leaf litter helps to raise the soil pH of boreal forest, which is typically acidic and thus improves the soil biota (Suominen et al. 2003)

Aspen had little economic importance in previous time (1950-1960s) as it was only used in match industries, even it was systematically removed from cultivated forests in Finland, as it acts as an intermediate host for *Melampsora pinitorqua*, which cause rust disease to young Scots pines (Kouki et al. 2004). However, the economic interest started to increase in 1990s with the advancement in the paper industries. In recent times, aspen are primarily cultivated for the paper, pulp and plywood industries along with bioenergy production (Rytter 2006). In addition, *Populus* species are capable of growing well in moderately polluted soils and can efficiently remove soil pollutants such as cadmium and zinc (Hermle et al. 2006, Hassinen et al. 2009), and thus can be used for phytoremediation.

Apart from faster growth, hybrid aspen stem wood is characterized by a relatively high cellulose and low lignin concentration, confirming its suitability for biotechnology, pulpwood and energy production (Tullus et al. 2010). Another interesting feature of hybrid aspen is that they are able to cross with European aspen in nature and that backcross results in producing viable seeds and early competition. It has been predicted that in Finland the changing climate will benefit broadleaved trees such as birch (*Betula* sp.) and aspen over conifers (Kellomäki et al. 1996). In general, any species with high genetic diversity and phenotypic plasticity make them capable to adapt environmental changes (Jump et al. 2009, Grulke 2010), and thus increase the importance of that particular species.

It is well documented that the *Populus* species possess significant clonal differences for a number of attributes such as growth rates and productivity, individual leaf area, leaf growth morphology, internal leaf morphology, stomatal morphology and their movement, and photosynthetic capacity (Barigah et al. 1994, Yu et al. 2001, Marron et al. 2005). Moreover, in different environmental condition such as soil properties, genotypic variations are often observable within *Populus* species (Yu and Pulkkinen 2003).

2.1.3 Importance as a model tree

The economic and ecological importance of forest trees are the driving force for developing model systems to study tree physiology and biology. So far, as a model plant, Arabidopsis has been used widely as many aspects of plant biology are similar including the trees. However, it is important to study some unique anatomical and physiological features, for example leaf and flower phenology and seasonal reallocation of nutrients in trees themselves, which are largely related to their perennial growth pattern. *Populus* species are one of the solution as in forest genetics they have been accepted by tree physiologists as a model system. In forest biotechnology, the first studied tree genus is *Populus* (Strauss et al. 2004). In 1990s, two books on poplar biology has been published, reviewing and supporting the strength of poplar as model forest tree (Bradshaw 1996, Klopfenstein et al. 1997). Wide genetic diversity, large distribution area and significant genetic polymorphisms in natural population of *Populus* offer scientists a profound basis for studies in tree morphology, anatomy, physiology and response to biotic and abiotic stress (Farmer Jr 1996). Relatively small genome size, that has already been sequenced for *P. trichocarpa* is another important feature in *Populus.* *Populus* haploid genome size (five hundred and fifty million base pairs) is only four times larger than in *Arabidopsis* and forty times smaller than the genome size of conifers, for example loblolly pine (Bradshaw Jr and Stettler 1993). For molecular genetic studies, *Populus* has several important features that made it ideal for gene transfer including ease of regeneration *in vitro* and possibility of genetic transformation using *Agrobacterium* vector system. There are several documentation about genetic modifications in *Populus*, for example around thirty years ago in USA a

herbicide-tolerant gene was transferred to *Populus* (Fillatti et al. 1987). The first two commercial transgenic *Populus* species producing Bt (*Bacillus thuringiensis*) -toxin and a combination of Bt-toxin and proteinase inhibitor (to gain resistance against leaf feeding insect) was released in China in year 2002 and established for a commercial plantation in year 2003 (Lida et al. 2003, Valenzuela et al. 2006).

2.2.1 Role of Stomata

Stomata and the impervious cuticle surface on leaves are important contributors to speciation and evolutionary changes as stomatal control of water loss facilitate terrestrial plants to occupy habitats with changing environmental conditions (Raven 2002). Stomatal pores are the gateways for gaseous exchange (most importantly water and CO_2) between leaf interior and atmosphere. The total stomatal pore area is considered only five percent of the leaf surface. However, without cuticle, a leaf might loss seventy percent of its water content (Hetherington and Woodward 2003).

Minimizing transpirational water loss during CO_2 uptake is the major role of stomata (Berry et al. 2010). In order to avoid wilting and to drive growth by uptaking enough CO_2, it is essential to limit water loss under such condition where water is less available, for example; under drought condition. One interesting phenomenon of stomata is that they can balance between water loss prevention and CO_2 uptake, depending on the environment plants are growing. Plants growing under abundant water supply or lower evaporative demand, stomatal movement shifts towards maximizing CO_2 uptake or evaporative cooling at high temperature. On the other hand, plants growing under lower water supply condition, the priority moves towards increasing water use efficiency (Lu et al. 1998, Hetherington and Woodward 2003).

2.2.2 Molecular mechanism of stomatal movements

Stomatal opening and closing is regulated through changes in turgor pressure of the guard cells. Stomata open with the increase of osmotically active solutes content in the guard cell resulting in water uptake and thus increasing in the guard

cell turgor. In contrast, stomata close with the flow out of the solutes causing water efflux, thereby, decreasing guard cell turgor. The uptake and intake of the solutes take place through ion channels and ion transporters, present on the plasma membrane, as the plasmodesmata is absent in mature guard cells. Ion transporters consist of different pumps, carriers, symporters and antiporters; that drive transport against a free energy gradient using energy from ATP. Ion channels are proteins that facilitate active movement of ion fluxes through a controlled proteinaceous aperture (Pandey et al. 2007).

Extensive studies have been performed on the molecular mechanism of stomatal regulation. During light-induced stomatal opening, the plasma membrane hyperpolarize through activation of H^+-ATPase leading to efflux of cytosolic Hydrogen ion (H^+). This event leads to activation of inward rectifying Potassium ion (K^+) channel causing K^+ influx (Pandey et al. 2007, Kinoshita and Hayashi 2011). On the contrary, depolarization of plasma membrane, in response to high CO_2 concentration, O_3 and absisic acid (ABA) leads to stomatal closing. Membrane depolarization is mediated by anion channel activation and inhibition of H^+-ATPase activity (Keller et al. 1989, Schroeder and Hagiwara 1989). Anion channel activates with the increase of cytosolic calcium ion (Ca^{2+}) concentration that causes membrane depolarization in guard cells that exert a driving force for K^+ efflux through outwardly rectifying K^+ channels (Schroeder et al. 1987). The efflux of both anions, i.e. Chloride (Cl^-), malate^{2-}, Nitrate (NO_3^-) and K^+ causes loss of guard cells turgor and leads to stomatal closure (Schroeder and Hagiwara 1989, MacRobbie 2006, Vahisalu et al. 2008, Kim et al. 2010, Negi et al. 2014). Thus stomatal movement depends on the changes in turgor, stomata open and close with the increase and decrease in turgor of guard cells respectively.

Plant growth, development and physiology is affected by the quality and quantity of light. Several important processes of plant including photosynthesis, stomatal regulation, leaf expansion and senescence, stem elongation, seed germination and dormancy are depending on light. Stomata open in response to light and allow CO_2 uptake for photosynthesis. On the other hand, stomata close in absence of light. Different kinds and wavelengths of light regulate stomata differently, for example, experiment conducted on maize, blue light has shown to be more

efficient in driving stomatal opening than red light (Vavasseur et al. 1990). K+ and Cl- uptake, malate synthesis and starch hydrolysis is stimulated by blue light, whereas in the absence of K^+ uptake sucrose accumulation is stimulated by red light during photosynthesis (Zeiger et al. 2002).

2.3.1 Types of anion channel and their role

Anion channels are proposed to have a central role in a large number of cellular processes such as signal transduction, ion transport, growth control and guard cell volume regulation (Schmidt and Schroeder 1994). They represent a large class with highly diversified properties. Different anion channels have different anion selectivity and depending on the selectivity they plays particular roles, for example Cl^--selective channels are involved in salt tolerance (Jossier et al. 2010), nitrogen homeostasis is maintained by NO_3^--selective channels and organic acid selective channels are involved in carbon metabolism or pH regulation (Meyer et al. 2010). Plant cell cannot survive with constantly open anion channels as it would cause a massive loss of ions and depolarization (Kollist et al. 2011).

Patch-clamp experiment on guard cell plasma membrane of *Vicia faba* have identified and characterized two types of anion channels; slow-type (S-type) (Schroeder and Hagiwara 1989) and rapid-type (R-type) (Hedrich et al. 1990). R-type anion channel is voltage dependent and it takes milliseconds to activate or deactivate. R-type currents also exhibit time dependent inactivation in *V. faba*, where S-type anion channel activity exhibit weak voltage dependency and do not inactivate with time (Kollist et al. 2011). These two types of anion channels play a role in stomatal closure, but the response is not equal. For example both the R-type and S-type channels participate in ABA-induced stomatal closure (Roelfsema et al. 2004), but in response to increase in partial pressure of CO_2, only S-type anion channel is activated consistently, whereas the R-type channel is either activated or inactivated (Raschke et al. 2003).

Both S-type and R-type anion channels are proposed to be permeable to different anions like $malate^{2-}$, NO_3^- and Cl^- (Schmidt and Schroeder 1994, Roelfsema et al. 2012). S-type anion channel is activated in response to increased CO_2 concentration, darkness and O_3 via phosphorylation (Schmidt et al. 1995, Brearley

et al. 1997, Vahisalu et al. 2008, Vahisalu et al. 2010). ABA has been shown to activate S-type anion channel through elevation of free cytosolic Ca^{2+} (Schroeder and Hagiwara 1989, Hedrich et al. 1990).

Though anion channels and their regulatory mechanisms have been studied and characterized long before, a gene named *SLAC1* encoding S-type anion channels involved in stomatal closure was identified relatively recently (Negi et al. 2008, Vahisalu et al. 2008). It has been shown that guard cells isolated from *Arabidopsis thaliana slac1* mutants have impaired Ca^{2+}- and ABA-induced S-type anion channel activity (Vahisalu et al. 2008). Thus, the studies provide strong genetic evidence for the model that S-type anion channels play the most crucial role in signal-induced stomatal closing (Schroeder and Hagiwara 1989).

Studies have shown that the CO_2 (HCO_3^-)-dependent activation of S-type anion channels depend on OPEN STOMATA 1 (OST1) protein kinase (Xue et al. 2011) that phosphorylate and activate the guard cell anion channel SLAC1 (Geiger et al. 2009), suggesting that high CO_2 concentrations stimulate OST1, and results in activation of SLAC1. OST1 also play crucial role in ABA dependent stomatal closure (Mustilli et al. 2002) by activating S-type anion channel SLAC1 through phosphorylation of its N terminus (Geiger et al. 2009, Vahisalu et al. 2010).

2.3.2 Structure of SLAC1

SLAC1 is a general regulator in guard cells and is play the most crucial role in stomatal closure in response to elevated CO_2, O_3, ABA, H_2O_2, extracellular Ca^{2+}, absence of light and humidity (Negi et al. 2008, Vahisalu et al. 2008). Different studies provided the evidenced that SLAC1 expression is highly specific for guard cells (Negi et al. 2008, Imai et al. 2015).

Based on homology with the *Haemophilus influenza* Teha protein, a three dimensional structure of SLAC1 has recently been predicted (Chen et al. 2010). *SLAC1* represent the founder member of a small gene family comprised of *SLAC1* and four *SLAC1*-homologs *SLAH1-4* in *Arabidopsis* (Negi et al. 2008, Chen et al. 2010). The *SLAC1* gene encodes a membrane protein consisting of ten predicted transmembrane α-helices with a large N- and C-terminal domain arranged on a

C4-dicarboxylate transporter/malic acid transport domain (Pfam PF03595). The *SLAC1* protein orthologues contains four conserved phosphorylation sites at the N-terminal tail and a conserved phenylalanine 450. This phenylalanine 450 residue has been shown to be essential for the function of the anion channel by changing its orientation (Chen et al. 2010). However, study based on genetic evidence of hybrid aspen, conducted by Sanna Ehonen in Jaakko Kangasjärvi's lab (Division of Plant biology, Department of Bioscience, University of Helsinki, personal communication), found that most of the functional elements are absent in *Populus* SLAC1. The mRNA of two different alleles, 'SLAC1 small' and 'SLAC1 big' has been sequenced from hybrid aspen clone 51. The studied structure of various protein models based on the genomic sequence of *P. trichocarpa* showed that *Populus* SLAC1 lacks the conserved phosphorylation sites at the N-terminal, the key amino acid residue phenylalanine 450 and most of the transmembrane domain (Figure 1, Figure courtesy Sanna Ehonen, University of Helsinki) suggesting that Populus species may not have functional SLAC1 anion channel (Sanna Ehonen, University of Helsinki, personal communication).

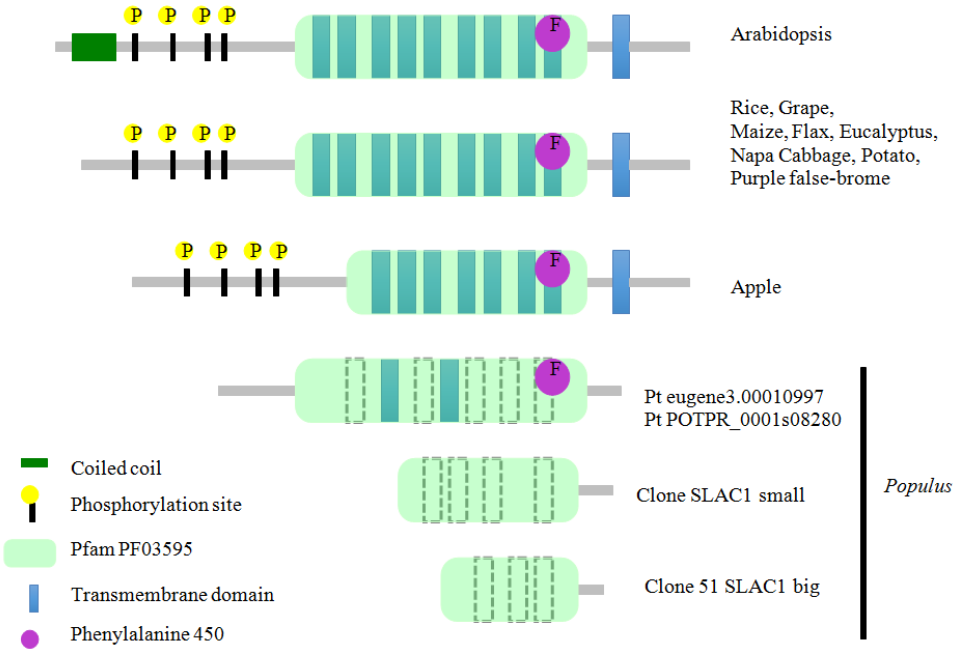

Figure 1. Protein structures of SLAC1 orthologues of different species. With the exception of *Populus* The SLAC1 protein orthologues of all studied species contains four conserved phosphorylation sites at the N-terminal and a conserved key amino acid residue phenylalanine 450 in the Pfam PF03595 domain. In addition, in the N-terminal there is a coiled coil domain in Arabidopsis SLAC1 (Figure courtesy; Sanna Ehonen, University of Helsinki, personal communication)

3 AIM OF THE STUDY

The primary aims were to select a guard cell specific promoter that will drive the expression of Arabidopsis *SLAC1* predominantly in the guard cells. Experiments were performed to observe if there would be any influence of *AtSLAC1* expression in growth of transgenic lines compared to wild type and to see the expression level of *AtSLAC1* in different transgenic lines. In addition, this study was conducted to investigate how the transgene (*AtSLAC1*) affect the rapid stomatal closure in hybrid aspen since *SLAC1* in *Populus* is most likely nonfunctional. More precisely the objective was to investigate whether the transgenic lines show a clear response to elevated CO_2 concentration by closing the stomata rapidly, thus to identify the strongest transgenic lines, that showed rapid stomatal closure in response to elevated CO_2 concentration for further field experiment. To achieve this aim, a set of different experiments such as Growth and water loss measurements and Gas Exchange measurements were conducted.

4 MATERIALS AND METHODS

4.1 Plant materials

Transgenic hybrid aspen clone 51 was used as plant material. Different transgenic hybrid aspen lines expressing *Arabidopsis thaliana SLAC1* (at1g12480) gene, were made. The transgene construct was containing *promoter::AtSLAC1* with either Human influenza hemagglutinin (HA) or Enhanced Yellow Fluorescent Protein (EYFP) tag. Two guard cells-specific promoters, *pSLAC1* (1504 bp, *Arabidopsis thaliana*) and *pGC1* (1987 bp, *P. trichocarpa*) were used. Hybrid aspen were transformed with *pSLAC1::AtSLAC1-HA* and *pGC1::AtSLAC1-HA* construct using pGWB13 destination vector (Fig. 2A, figure courtesy Sanna Ehonen, University of Helsinki), and pGWB40 destination vector (Fig. 2B, figure courtesy Sanna Ehonen, University of Helsinki) was used for *pSLAC1::AtSLAC1-EYFP* and *pGC1::AtSLAC1-EYFP* constructs (Nakagawa et al. 2007). Genetic transformation of hybrid aspen was conducted using *Agrobacterium tumefaciens* strain GV3101 (pMP90). Genetic transformation was done as previously described (Häggman et al. 2003). For promoter-driven GUS expression lines (*pSLAC1::GUS* and *pGC1::GUS*), promoters were cloned into the pMDC162 destination vector (Curtis and Grossniklaus 2003).

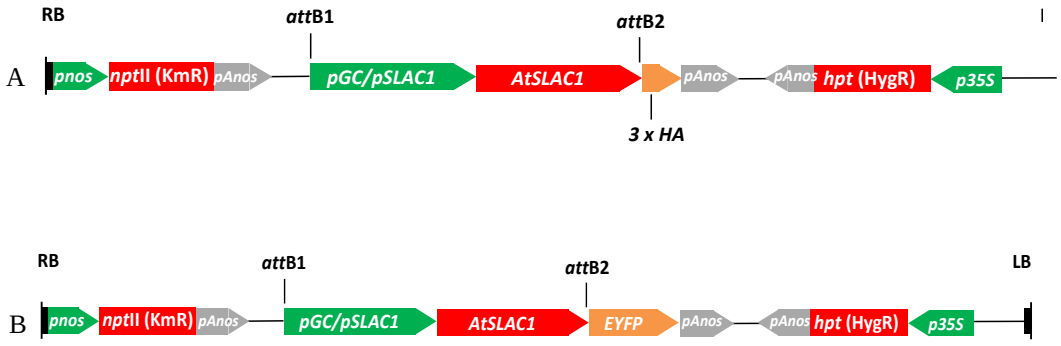

Figure 2. Schematic presentation of destination vector pGWB13 (A) with either *GC1* or *SLAC1* promoter, *AtSLAC1* and HA tag. Schematic presentation of destination vector pGWB40 (B), with either *GC1* or *SLAC1* promoter, *AtSLAC1* and EYFP tag. (Figure courtesy: Sanna Ehonen, University of Helsinki, personal communication)

4.2 Growth measurement

Growth measurement was performed with the greenhouse-grown control and tagged lines (either HA or EYFP) only. Shoot height was measured from the top of the soil to apical buds. The measurements were done in approximately every two weeks, starting from three weeks after transplanting the seedlings in the greenhouse until the plants were eleven-weeks-old. Four biological replicates were used for the measurement of each individual transgenic lines and ten biological replicates were used for control. When the plants were eleven weeks old, stem diameter was measured at the height of approximately 10 cm from the soil top. As the stem was not exactly round in shape, at the corresponding position three technical repeats were made for each plant.

4.3 Water-loss measurements

Water loss experiment was performed with seven-weeks-old greenhouse-grown control and transgenic plants. Leaves from 7^{th} internode was used. The weight of detached leaves was measured at different time points, 30 minutes, 1 and 2 hour, and the water loss was expressed as the percentage of initial fresh weight. Four biological replicates from each independent lines were used during this experiment.

4.4 Plant genomic DNA isolation and confirmation of transgenic plant through PCR

In order to confirm that the transgenic lines were containing transgene, genomic DNA were isolated from *in vitro*-grown plant leaves using NucleoSpin® Plant II kit (MACHEREY-NAGEL) and following manufacturers' instruction. In brief, the *in vitro* grown leaf samples were collected and grinded using mortar and pestle in the presence of liquid nitrogen. During grinding, cautions were taken so that the samples do not thaw at any time. The samples were homogenized well to facilitate effective lysis procedure. Approximately 100 mg homogenized samples were taken and 400 µl of lysis buffer (PL1) was added then vortexed the mixture immediately. Then 10 µl of RNase A solution was mixed and incubated for 20 minutes at 65^0C. RNase A solution was added to get pure genomic DNA by removing RNA and to facilitate photometric quantification of pure sample. The

steps for lysate filtration, binding DNA to membrane washing and drying of the membrane was done following exact protocol. During elution 25 µl of elution buffer was used and it elution was performed only once. The concentration and purity of isolated DNA samples were measured by spectrophotometer (Nanodrop, Thermoscientific).

The transgenic lines were detected by PCR. Primer sequences for actin (used as a control) and kanamycin used for PCR amplification were:

Gene	Type	Primers sequence 5’ > 3’
ACTIN	Forward	CGATGCCGAGGATATTCAAC
	Reverse	ACCAGTGTGTCTTGGTCTACCC
KANAMYCIN	Forward	AGACAATCGGCTGCTCTGAT
	Reverse	AGCCAACGCTATGTCCTGAT

One µl (approximately 40 ng) of genomic DNA was used for PCR amplification. Twenty µl of final reaction volume was prepared for PCR reaction using following reagents:

Taq 10X buffer	2,00 µl
10 mM dNTP	0,40 µl
10 pmol/ µl forward primer (Kanamycin)	0,25 µl
10 pmol/ µl reverse primer (Kanamycin)	0,25 µl
10 pmol/ µl forward primer (Actin)	0,25 µl
10 pmol/ µl forward primer (Actin)	0,25 µl
Milli-Q water	15,52 µl
DNA template	1,00 µl
FIREPol® DNA polymerase (5 U/ µl)	0,08 µl
Final volume	20,00 µl

With the amount of reagent listed above a master mix was prepared according to the sample number and distributed 19 µl per PCR tube. One µl of DNA sample

was added before the PCR start. Finally, PCR reaction was performed according to following program:

1. 95°C for 5 minutes
2. 95°C for 30 seconds
3. 60°C for 30 seconds
4. 72°C for 40 seconds
5. go to 2 for 35 X
6. 72°C for 5 minutes
7. 8°C forever

The annealing temperature maintained as 2-6°C lower than primer melting temperature and~1 min/kb was used as the elongation time (Solis BioDyne). After PCR the amplified fragments were checked in agarose gel for expected product size.

4.5 Histochemical GUS assays

Localization of histochemical GUS activity was investigated in samples collected from transgenic lines (*pSLAC1::GUS* and *pGC1::GUS*). Leaf samples were collected at different age of plants (10 days, 5, 9 and 11 weeks; leaf samples were from 4th, 8th, 18th and 23rd internode respectively). Immediately after collecting the samples they were immersed in ice cold fixation solution (90% acetone) for 1 hour. Then the fixation solution was removed and washed twice with 1X NaP buffer each for 30 minutes. The samples were kept on a shaker during washing steps to facilitate complete removal of fixation solution. During first wash only 1X NaP buffer was used but on the next wash Ferrocyanide was added with 1X NaP buffer at a concentration of 1.5 mM. This was done to increase the cell specificity and to decrease the diffusion of the GUS staining. After second wash the buffer was removed and previously prepared GUS staining solution was added. Then vacuum infiltration was done for 2 minutes to increase the penetration of the solution inside the sample. Vacuum infiltration was done in dark as 5-bromo-4-chloro-3-indolyl-b-D-glucuronide cyclohexyl-ammonium salt (X-Gluc) is light-sensitive and the pressure was released gently. After that the samples were stained in dark at 37°C for approximately 17 hours. Once the

staining was completed, the staining solution was removed and washed twice with Milli-Q water. Before analyzing the samples under microscope, the samples were destained by adding absolute ethanol and incubating them at 65°C for 30 min. After that destaining solution was removed, 30% glycerol was added to each samples and stored for further analysis. Gus staining was observed using light microscope (Leica) and photos were taken with a camera attached to it.

The recipes for GUS staining solution and buffers used during this experiment are as follows:

Solutions:

Fixation solution: 90% Acetone

1x NaP: 0,05M Sodium phosphate buffer, pH 7.2

GUS staining solution (for 500 ml):

15 ml 1M Na2HPO4

10 ml 1M NaH2PO4

0,2469 g K4Fe(CN)6 (1,5mM)

0,3167 g K3Fe(CN)6 (1,5mM)

250 mg X-Gluc dissolved in 1 ml DMF (dimethylformamide)

500 µl Triton X-100

Milli-Q water to make final volume 500 ml

GUS staining solution was stored at -20° C.

4.6 RNA extraction

Leaf samples were collected for RNA extraction from greenhouse grown transgenic lines (*pSLAC1::AtSLAC1-HA*, *pSLAC1::AtSLAC1-EYFP*, *pGC1::AtSLAC1-HA* and *pGC1::AtSLAC1-HA*). The plants were 3 week-old after transplanting in the mini green house and leaves from 5th internode were collected. The samples were kept in liquid nitrogen immediately after collection to avoid RNA degradation. Total RNA extraction was done using Spectrum™ Plant Total RNA Kit (SIGMA-ALDRICH©) and following manufacturers' instruction. In brief, collected leaf samples were grinded with mortar and pestle to fine powder submerging them in liquid nitrogen. During sample grinding the samples

and the mortar and pestle kept frozen at all the time. 100 mg of frozen tissue powder was used for total RNA extraction. Lysis solution /2-mercaptoethanol (2-ME) mixture was prepared as recommended by manufacturer (10 µl of 2-ME for each 1 ml of lysis solution) and every time fresh lysis solution/2-ME mixture was used. During lysis step 500 µl of lysis solution/2-ME mixture was added to each solution and vortexed vigorously to mix the powder evenly in the solution. Then the samples were incubated at 56°C for 5 minutes followed by centrifugation at 14,000 X g for 3 minutes to pellet the cell debris. The lysate supernatant then pipetted into filtration column assembled into a 2 ml collection tube and centrifuged at 14,000 X g for 1 minute and the flow-through lysate was collected. During RNA binding step 500 µl of binding solution was added to collected flow through lysate, mixed immediately and thoroughly by pipetting up and down for at least 5 times. Then the mixture was transferred to a binding column, centrifuged at 14,000 X g for 1 minute and discarded the flow-through. 3 consecutive wash was done using the wash buffer and instruction provided followed by an additional centrifugation at 14,000 X g for 1 minute to dry the binding column. Elution was done only once using 50 µl of sterilized water. Extra caution was taken during all the steps to avoid introducing exogenous RNases, especially during final wash and elution steps. The working area kept clean and sterilized, clean pipette tips were used and gloves were changed frequently. The concentration and purity of extracted RNA was determined by spectrophotometry (Nanodrop, Thermo Scientific). The samples were then stored at -70°C for further analysis. Before storing 1 µl of water containing 0,1 µl of Ribolok™ Ribonuclease inhibitor (Thermo Scientific Fermentas) was added to each sample.

4.7 cDNA synthesis

cDNA synthesis was done using 3 µg of total RNA. In brief, Sterilized water was added to each sample to make the final volume 16.8 µl. Dnase treatment was performed by adding 3.25 µl of DNaseI mix and incubating at 37°C for 30 minutes. After incubation 2 µl of 50 mM EDTA was added followed by incubating the mixture for 10 minutes at 65°C in order to inactivate DNaseI and to denaturate RNA. Reverse transcription (RT) was completed by adding 9.5 µl of RT mix containing RevertAid Premium RT, Ribolock Ribonuclease inhibitor (Thermo Scientific Fermentas) to the treated samples, keeping them on ice. The

samples then incubated for 2 hours at 50°C followed by inactivation of RevertAid Premium through incubation for 5 minutes at 85°C. The synthesized cDNA was ready to use and it was diluted to 100 µl before use.

Recipes for DNaseI mixture and RT mixture are as follows:

DNase mix		RT mix	
10x buffer	2,00 µl	Oligo-dT (20)	1,00 µl
DNaseI	1,00 µl	5X buffer	6,20 µl
Ribolock RNase inhibitor	0,25 µl	10 mM dNTP mix	1,50 µl
		RevertAid Premium	0,50 µl
		Ribolock RNase inhibitor	0,25 µl
Total	3,25 µl	Total	9,45 µl

4.8 Gene expression analysis through qPCR

To study the gene expression, qPCR was performed with 1 µl of cDNA template with 5X HOT FIREPol EvaGreen qPCR mix and specific primers. At first master mix was prepared for each gene of interest and for the reference genes. The master mix was vortexed briefly to mix all the ingredients properly. Then 10 µl of reaction mix including cDNA template was pipetted in each well of a 384 wells plate. All the steps were done on ice to avoid evaporation of the reaction volumes. Clean pipettes and pipette tips were used. Once the plate was ready, the reactions were centrifuged down. Three technical repeats for each biological samples were made and the plate was run in Bio-Rad CFX 384 PCR machine. The cycle conditions were 1 cycle initiating with 95°C for 10 minutes, 39 cycles with 95°C for 15 seconds, 60°C for 30 seconds, 72°C for 30 seconds. At the end of each run a melting curve was generated for each sample. To ensure the purity of amplified product, melting curve and melting temperature were checked carefully. After the run was completed the raw cycle threshold values were exported and analyzed in qBase 2 (Biogazelle, Hellemans et al., 2007) using three reference genes, *TIP41-like* (Potri.009G093200; 119 bp mRNA), *TUBULIN* (Potri.006G095000; 222 bp mRNA), and *ACTIN 1* (Potri.001G309500; 127 bp mRNA).

Primer sequences were:

Gene	Type	Primers' sequence 5' > 3'
AtSLAC1	Forward	CGGGCTCTAGCACTCACTC
	Reverse	GCAAGATCGTTTGGGAACAA
TIP41-like	Forward	GCTGCACTTGCATCAAAAGA
	Reverse	GCAACTTGGCATGACTCTCA
TUBULIN	Forward	GATGCTTACCTTCTCCGTCTTTCCC
	Reverse	GTGACCCCAGACATTGTAGCAGAAA
ACTIN 1	Forward	CGATGCCGAGGATATTCAAC
	Reverse	ACCAGTGTGTCTTGGTCTACCC

The recipes for qPCR ingredients are as follows

3 times master mix	
5X HOT FIREPol EvaGreen mix	6 µl
5 µM gene specific forward + reverse primer	1,5 µl
cDNA template	3 µl
Sterilized water	20,5 µl
Total	31 µl

4.9 Gas exchange measurements

Gas exchange measurements were performed with greenhouse-grown control and transgenic lines using a portable gas exchange fluorescence system (GFS-3000, Walz, Germany). The experiment was performed with eight-week-old plants and leaves from 13^{th}-15^{th} internode from the shoot apex were used. The measurements were conducted during 5^{th} July – 3^{rd} August 2014 in between 9.00 h and 17.00 h. The response of stomatal conductance to elevated CO_2 concentration was measured by increasing CO_2 from 200 ppm to 1000 ppm at a constant light intensity (1000 µmol m^{-2} s^{-1}), cuvette temperature was 22°C and relative humidity

(RH) was 60%. The flow rate (650 µmol s^{-1}) was also constant for whole gas exchange experiment and the area of the leaves inside the cuvette was 8 cm^2.

4.10 Statistical analysis

The growth measurement and water loss measurement data were analyzed using the statistical package SPSS (version 22.0, SPSS Inc., Chicago, IL, USA). Prior to analysis normality of the data were checked by Shapiro-Wilk's test. Analysis of variance (ANOVA) was used to compare the mean and the differences were assessed by Tukey's multiple comparison test. The threshold of significance was set to $P = 0.05$. Data from qPCR were analyzed using qBase2 (Biogazelle) software.

5 RESULTS

5.1 PCR analysis of transgenic lines

In order to check that all the lines were transgenic, PCR was performed with isolated genomic DNA from both transformed and untransformed control lines. After that the amplified products were checked in agarose gel. Two different primer pairs (Actin and Kanamycin) were used for this purpose and it was expected that the untransformed lines (HH51) will produce only one band at 225 base pair (bp) for actin while the transgenic lines will produce an additional band of 593 bp for kanamycin. All transgenic lines tested by PCR showed positive except for line number 6 and 11 for the construct *pGC1::AtSLAC1-HA.* However, for these two lines the band corresponding to actin is also missing, indicating that the DNA quality was most likely bad and thus there were no bands.

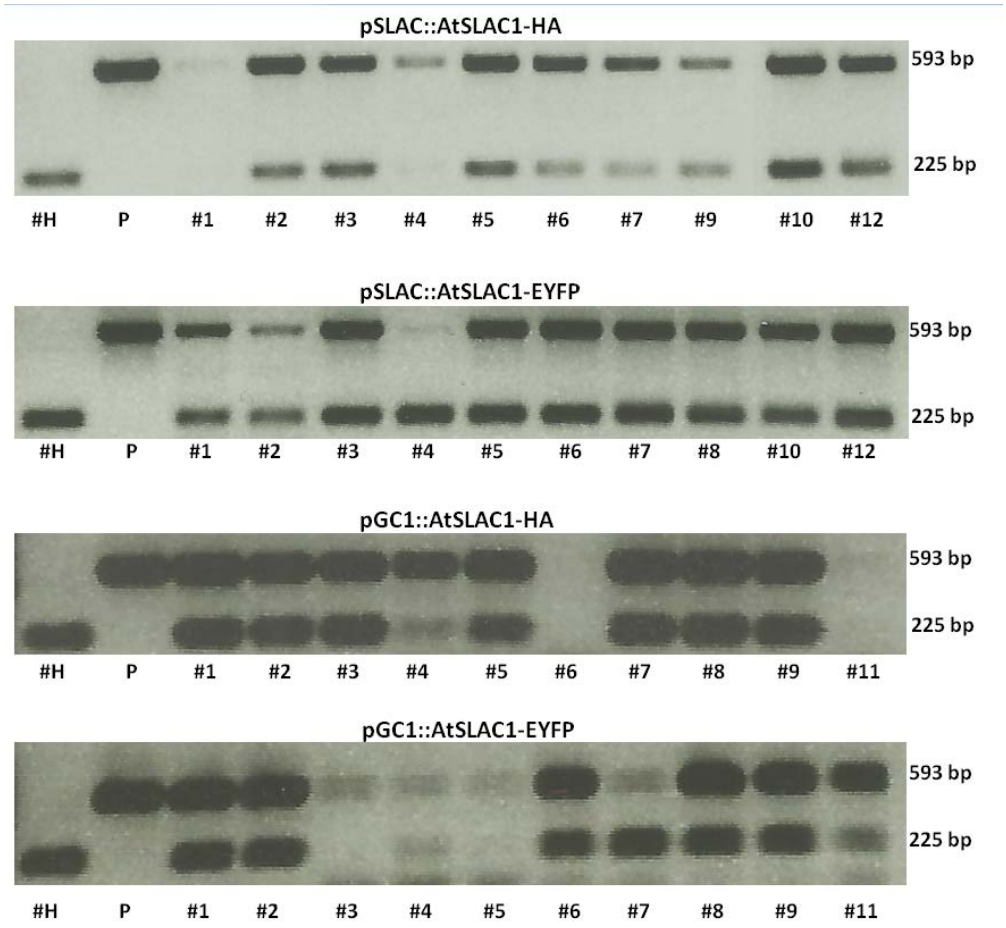

Figure 3. Confirmation of transgenic lines by PCR. Two primer pairs, actin and kanamycin were used. Here #H stands for untransformed control lines (HH51), P is positive control for transgenic hybrid aspen lines and # followed by different numbers indicate independent lines form corresponding genotypes. Transgenic lines produced one additional band for kanamycin at 593 bp compared to control line (HH51) that produce only one band for actin at 225 bp position.

5.2 Growth and water loss

In order to see any differences between wild type and transgenic lines in two different growth parameters, height and basal diameter, as well as in water loss, analysis of variance (ANOVA) was used to calculate the mean difference and standard deviation followed by Tukey's multiple comparison test. The results are presented in Table 1 and Table 2. ANOVA showed significant differences in height and basal diameter for the transgenic lines of *pSLAC1::AtSLAC1-HA* and wild type (HH51) (Table 1, P <0.001 and P = 0.014 respectively). However, in Tukey's multiple comparison test showed only significant differences in height increment percentage for line 2, 4, 6 and 7 of *pSLAC1::AtSLAC1-HA* compared to wild type. Except for line 3, all the other lines showed higher percentage of height increment, compared to wild type. On the other hand, the basal diameter was lower in most of the lines (except line 1 and 7), compared to wild type, though the differences were very low. The differences were not significant in loss of initial fresh weight for *pSLAC1::AtSLAC1-HA* and wild type after two hours (Table 1, *P* = 0.256). Most of the independent lines of this genotype, compared to wild type plants, exhibited lower percentage of initial fresh weight meaning higher water loss (exception in lines 7 and 9).

The percentage of height increment and basal diameter differed significantly in wild type and independent lines of transgenic plants, *pSLAC1::AtSLAC1-EYFP* (Table 1, *P* = 0.010 and *P* = 0.002 respectively). The percentage of height increment was higher compared to wild type, except for three lines (lines 1, 4 and 7). For initial fresh weight loss, there was no significant difference observed (Table 1, *P* = 0.509). However, the basal diameter and loss of initial fresh weight was lower for most of the transgenic lines compared to wild type (except line 1 and 6 for basal diameter, and line 4 and 6 for initial fresh weight loss).

Table 2 represent ANOVA for wild type and individual lines from transgenic plants of *pGC1::AtSLAC1-HA* and *pGC1::AtSLAC1-EYFP* genotype. Only height increment and basal diameter was measured for these transgenic lines. ANOVA suggested that there were significant differences for both growth parameters, height increment percentage and basal diameter, for *pGC1::AtSLAC1-HA* (Table 2, *P* = 0.001 and 0.016 respectively). For this genotype. lines 2 and 10 showed

significantly higher percentage of height increment. Apart from line 1, in lines 4 and 5 height increment was marginally higher and basal diameter was slightly lower, except for lines 1, 7 and 10 compared to wild type.

For the genotype *pGC1::AtSLAC1-EYFP*, most of the transgenic lines (Table 2). (except for lines 1, 3 and 7), the height increment was little higher and basal diameter was marginally lower (excluding line 4) compared to wild type plants. Statistical analysis showed significant difference in height increment (Table 2, P = 0.012), but no individual lines were significantly different from wild types. The basal diameter was not significant (Table 2, P = 0.211) between the transgenic lines and wild type.

Table 1. Analysis of variance of average height, basal diameter and water loss for control (HH51) and different independent lines from the transgenic plants, *pSLAC1::AtSLAC1-HA* and *pSLAC1::AtSLAC1-EYFP*. Data in the table represent mean values (± SD). For control plants n = 10, for height measurement and basal diameter but for water loss measurement n = 7 and for transgenic lines n=4 in all the cases. In each row mean values followed by (*) are significantly different at the 5% level in Tukey's HSD test. *P* value indicate the level of significance at 5%.

	pSLAC1::AtSLAC1-HA											
	HH 51	# 1	# 2	# 3	# 4	# 5	# 6	# 7	# 9	# 10	# 12	*P*
Height increment (%)	88.70 ± 0.4	89.76 ± 1.08	90.45 ± 0.66*	88.39 ± 1.61	91.05 ± 0.37*	89.28 ± 0.5	90.25 ± 1.01*	90.73 ± 0.5*	88.93 ± 0.5	89.73 ± 0.55	90.10 ± 0.84	<0.001
Basal Diameter (mm)	11.84 ± 0.44	11.93 ± 0.41	11.26 ± 0.52	11.78 ± 0.91	11.08 ± 0.60	11.06 ± 0.44	10.82 ± 0.48	11.9 ± 0.63	11.54 ± 0.19	11.15 ± 0.39	11.77 ± 0.48	≤ 0.014
Loss of initial fresh weight (%)	72.9 ± 3.29	61.65 ± 18.53	60.54 ± 18.34	70.42 ± 1.97	70.51 ± 1.31	68.35 ± 2.42	67.66 ± 1.76	74.58 ± 1.30	72.21 ± 3.35	69.21 ± 0.89	71.87 ± 4.67	≤ 0256
	pSLAC1::AtSLAC1-EYFP											
	HH 51	# 1	# 2	# 3	# 4	# 5	# 6	# 7	# 8	# 10		*P*
Height increment (%)	88.7 ± 0.4	88.65 ± 0.40	90 ± 1.10	89.78 ± 0.77	87.45 ± 1.54	89.35 ± 0.69	89.06 ± 1.2	87.92 ± 0.10	88.95 ± 0.99	88.51 ± 0.98		≤ 0.010
Basal Diameter (mm)	11.84 ± 0.44	11.84 ± 0.45	11.74 ± 0.37	11.40 ± 0.28	11.53 ± 0.28	11.37 ± 0.55	12.85 ± 0.49	11.59 ± 0.50	11.48 ± 0.28	11.56 ± 0.63		0.002
Loss of initial fresh weight (%)	72.9 ± 3.29	66.96 ± 3.29	71.09 ± 2.73	65.18 ± 18.34	73.18 ± 2.34	70.18 ± 3.11	74.94 ± 1.99	71.49 ± 2.47	62.18 ± 19.21	69.64 ± 1.18		0.509

Table 2. Analysis of variance of average height and basal diameter for control (HH51) and different independent lines from the transgenic plants, *pGC1::AtSLAC1-HA* and *pGC1::AtSLAC1-EYFP*. Data in the table represent mean values (± SD). For control plants n = 10 and for transgenic lines n = 4. In each row mean values followed by (*) are significantly different at the 5% level in Tukey's HSD test. *P* value indicates the level of significance at 5%.

pGC1::AtSLAC1-HA											
	HH 51	# 1	# 2	# 4	# 5	# 6	# 7	# 8	# 9	# 10	*P*
Height increment (%)	887 ± 0.4	88.66 ± 0.40	89.44 ± 1.09*	88.38 ± 1.42	88.43 ± 0.43	89.33 ± 0.99	88.99 ± 0.55	90.19 ± 1.23	89.89 ± 0.63	90.66 ± 1.19*	≤ 0.001
Basal Diameter (mm)	1184 ± 0.44	11.99 ± 0.53	11.49 ± 0.63	11.70 ± 0.72	10.94 ± 0.32	11.53 ± 0.45	12.15 ± 0.42	10.90 ± 0.65	11.54 ± 0.72	11.93 ± 0.21	0.016
pGC1::AtSLAC1-EYFP											
	HH 51	# 1	# 2	# 3	# 4	# 5	# 6	# 7	# 8	# 9	# 11	*P*
Height increment (%)	8870 ± 0.4	88.55 ± 0.64	88.80 ± 0.86	88.68 ± 0.57	90.25 ± 0.83	89.12 ± 0.98	88.99 ± 0.56	87.99 ± 0.73	89.24 ± 1.33	90.12 ± 1.60	89.62 ± 0.76	0.012
Basal Diameter (mm)	1184 ± 0.44	11.58 ± 0.81	11.50 ± 0.58	11.25 ± 0.11	11.95 ± 0.54	11.06 ± 0.09	11.20 ± 0.52	11.69 ± 0.40	11.59 ± 0.54	11.43 ± 1.01	11.14 ± 0.34	0.211

5.3 Histochemical GUS assays

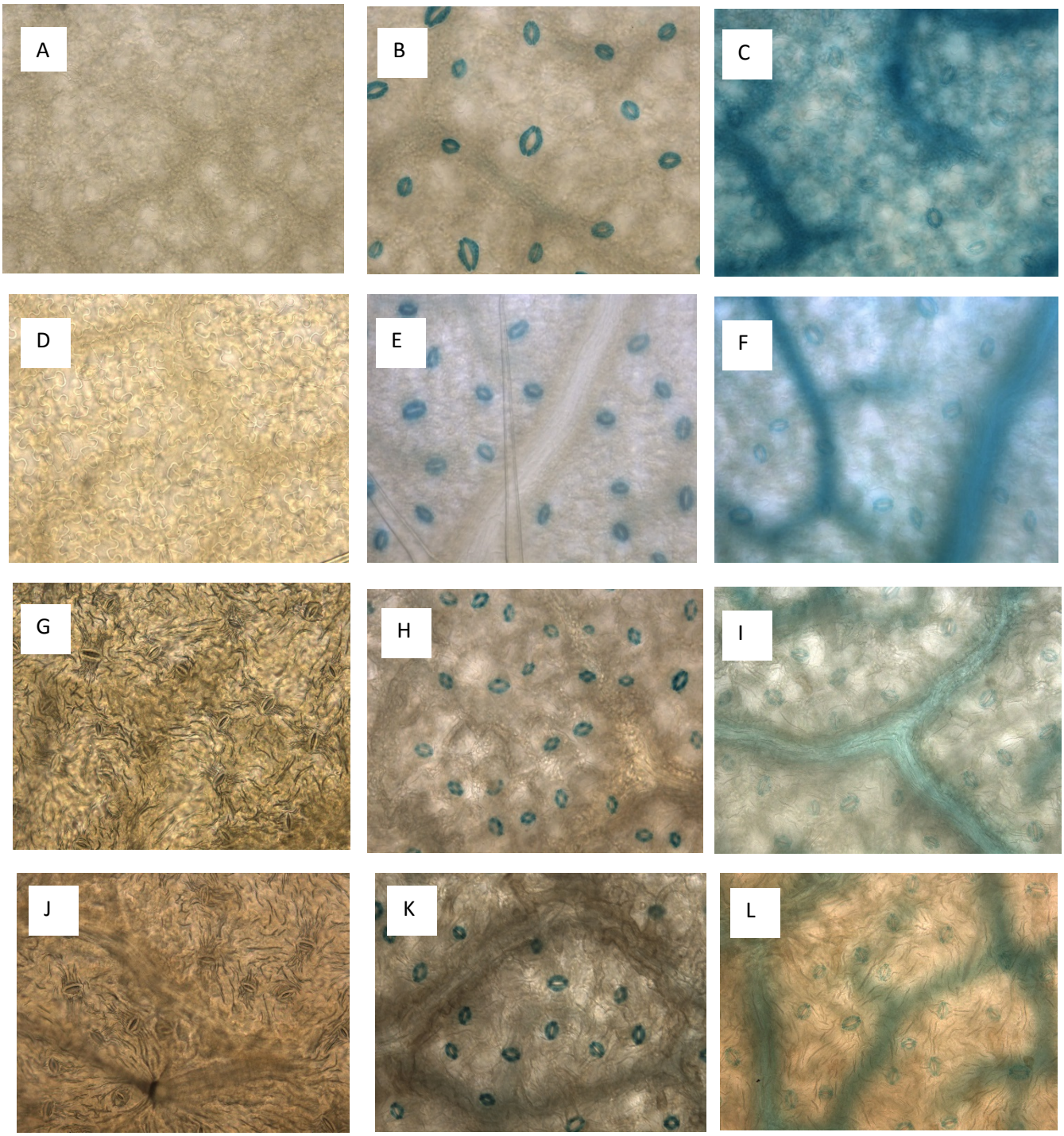

Figure 4: Expression pattern and histochemical localization of GUS activity in wild type (HH51) and transgenic plants. For wild type, the images are representative of six replicates, and for transgenic lines the images represent ten individual lines with three replicates of each. In wild type (4A, 4D, 4G and 4J, plants were 10 days, 5-, 9- and 11-week-old, respectively) there was no GUS activity observed as expected. For transgenic lines, the lines under control of *pSLAC1* (4B, 4E, 4H and 4K, plants were 10 days, 5 weeks, 9 weeks and 11 weeks old respectively) GUS activity was found to be specific to guard cell whereas transgenic lines carrying *pGC1* (4C, 4F, 4I, 4L, plants were 10 days, 5-, 9- and 11-week-old , respectively) showed weaker specificity to guard cell.

In order to investigate the tissue specific expression pattern of two promoters (*pSLAC1* and *pGC1*) and to select the guard cell specific promoter, histochemical GUS assays were performed in transgenic lines (*pSLAC1::GUS* and *pGC1::GUS*). The experiment was performed at different ages of greenhouse grown plants. Ten GUS lines with three replicates of each and six wild type plants were used. From the results (Fig. 4), it is clear that transgenic lines carrying the *pSLAC1::GUS* construct (Fig. 4B, 4E, 4H and 4K, 10 days, 5-, 9- and 11-week-old plants respectively), GUS activity was predominantly confined to guard cells regardless the age of the plants. Only a trace of GUS activity was detected in veins. On the other hand, GUS activity was not largely specific to guard cells for the transgenic lines carrying *pGC1::GUS* construct (Fig. 4C, 4F, 4I and 4L 10 days, 5-, 9- and 11-week-old plants, respectively) as GUS activity was found in veins, that indicated the *GC1* promoter has lower activity in guard cell compared to *SLAC1* promoter.

5.4 Stomatal regulation in response to elevated CO_2

To observe how the transgenic lines carrying Arabidopsis SLAC1 behave in response to elevated CO_2 concentration, stomatal conductance was measured. It was expected that with the elevated CO_2 concentration stomatal conductance would decrease faster in transgenic lines than in wild type plants since *Populus* SLAC1 is most likely nonfunctional. The result from stomatal conductance measurements showed that there was a decrease in stomatal conductance with elevated CO_2 concentration in transgenic plants compared to wild types. However, the result was not similar for all the biological replicates for each independent lines. In this result four independent lines with three biological replicates from each of the four genotypes, *pSLAC1::AtSLAC1-HA* (Fig. 5A), *pSLAC1::AtSLAC1-EYFP* (Fig. 5B), *pGC1::AtSLAC1-EYFP* (Fig. 6A) and *pGC1::AtSLAC1-HA* (Fig. 6B) are presented. Rest of the results are presented in appendix.

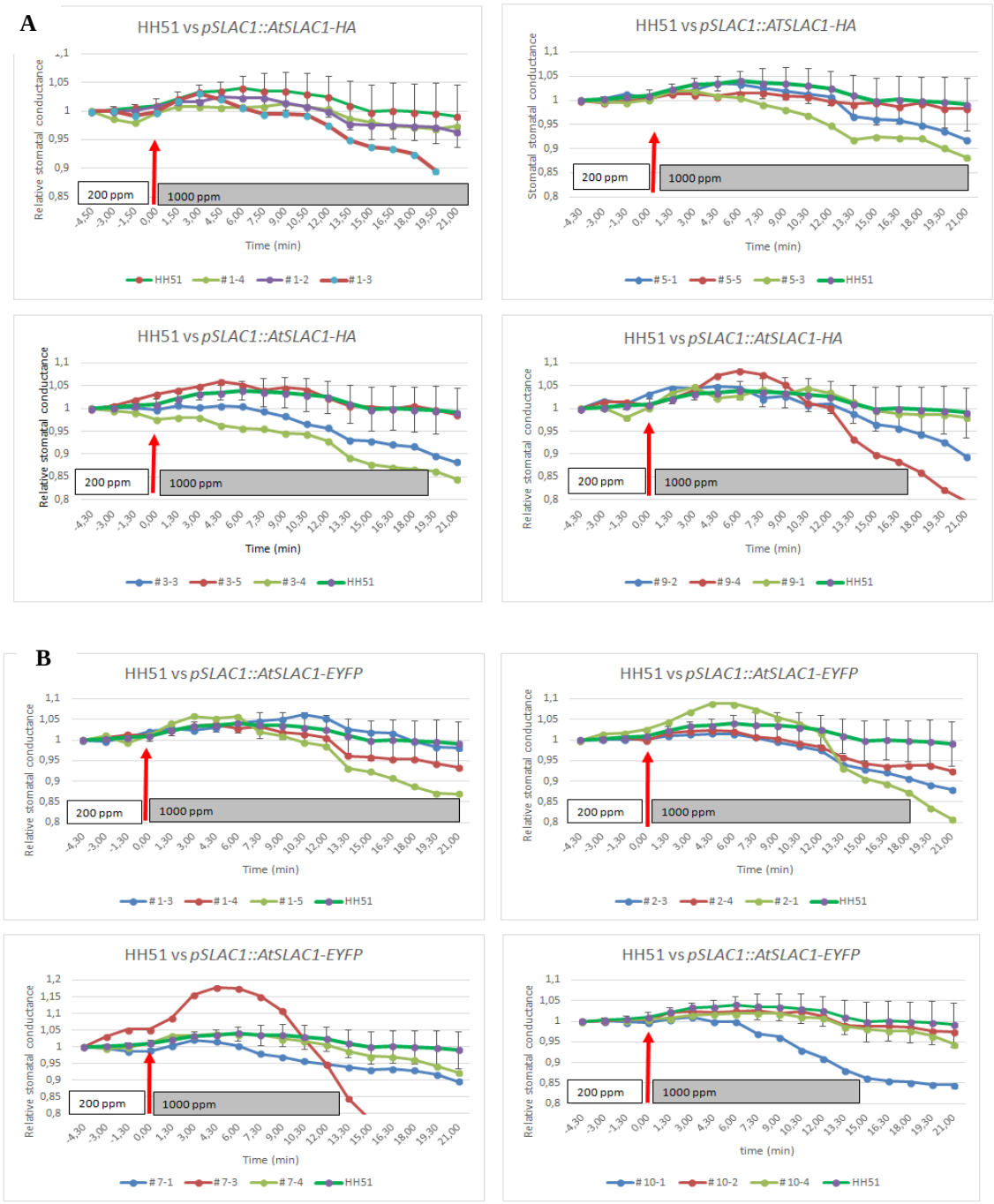

Figure 5. Relative stomatal conductance in response to changes in CO_2, in wild type (HH51) and transgenic lines, *pSLAC1::AtSLAC1-HA* (A) and *pSLAC1::AtSLAC1-HA* (B) . Data for wild type represent average of seven biological replicates ± SD.

All the biological replicates of individual lines (line 1, 3, 5 and 9) for the genotype *pSLAC1::AtSLAC1-HA* (Fig. 5A) showed a decrease in stomatal conductance compared to wild type after CO_2 elevation (from 200 ppm to 1000 ppm) , though the trend of decline was not the same. For example, as in control, the reduction of stomatal conductance was similar for the individual replicate 5 of line 3, replicate

5 of line 5 and replicate 2 of line 9 (Fig. 5A). Compared to wild type, *pSLAC1::AtSLAC1-EYFP* also showed similar response in stomatal behavior (Fig. 5B) in response to elevated CO_2. For some biological replicates (line 1-replicate 5, line 2-replicate 1, line 7-replicate 3 and line 10-replicate 1), the decline in conductance was sharp. On the other hand, some of the replicates (line 1-replicate 3, line 7-replicate 4, line 10- replicate 2 and line 10-replicate 4) showed the same response as in control.

Figure 6. Relative stomatal conductance in response to changes in CO2 in control (HH51) and transgenic lines, *pGC1::AtSLAC1-EYFP* (6A) and *pGC1::AtSLAC1-EYFP* (6B). Data for control represent average of seven biological replicates ± SD.

Lines from the genotypes *pGC1::AtSLAC1-EYFP* (Fig. 6A) and *pGC1::AtSLAC1-EYFP* (Fig. 6B) showed the same response as in *pSLAC1::AtSLAC1-HA* and *pSLAC1::AtSLAC1-EYFP*, meaning that some of the replicates responded in sharp decline of stomatal conductance, whereas, few of the replicates of the same line showed very little decline as in control plants.

5.4 qPCR Analysis

Finally, the transcript level of *SLAC1* was examined using qPCR. The result suggested that the amount of transcript abundances were not equal to each independent transgenic line. Some of the lines showed relatively high expression of *SLAC1*. For example, *pGC1::AtSLAC1-EYFP-4* (Fig. 7B) *pSLAC1::AtSLAC1-EYFP-2, -3* (Fig. 8B), *pSLAC1::AtSLAC1-HA-10, -3* (Fig.8A). On the other hand for *pGC1::AtSLAC1-EYFP-7* (Fig. 7B) *pSLAC1::AtSLAC1-EYFP-4, -5, -6, -7* (Fig. 8B), and *pSLAC1::AtSLAC1-HA-2, -5, -9* (Fig. 8A), the detected amount was very low.

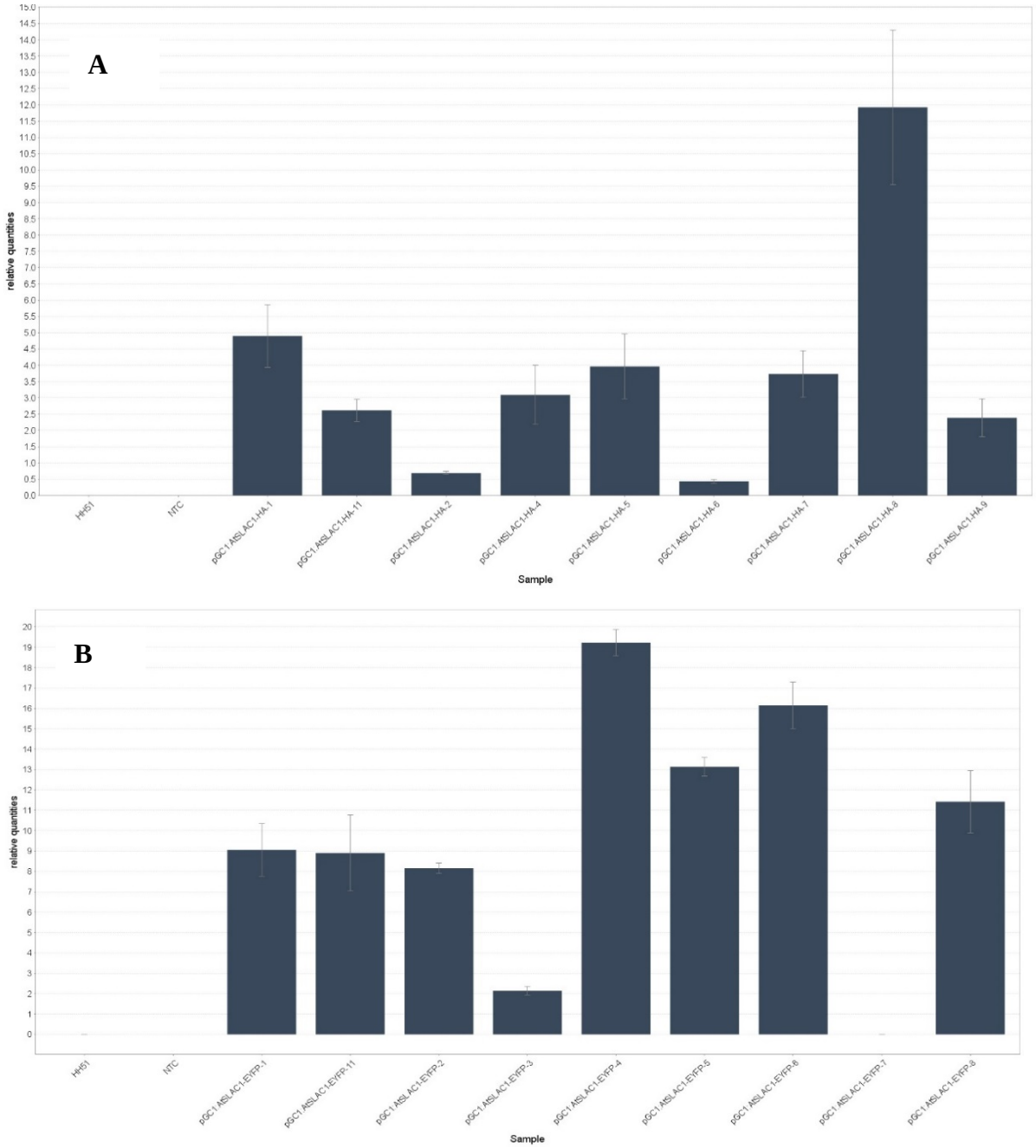

Figure 7: Expression of *SLAC1* in guard cells of transgenic lines containing *pGC1::AtSLAC1-HA* (A) and *pGC1::AtSLAC1-EYFP* (B) constructs. Transcript levels of SLAC1 was determined by qPCR. Three reference genes (TIP41-like, TUBULIN and ACTIN) were used for normalization. Data are the average of three biological replicates and for each replicate experiments were performed in triplicate. Bars represent ± SE for biological replicates. HH51 denotes control (untransformed) and NTC stands for non-template control.

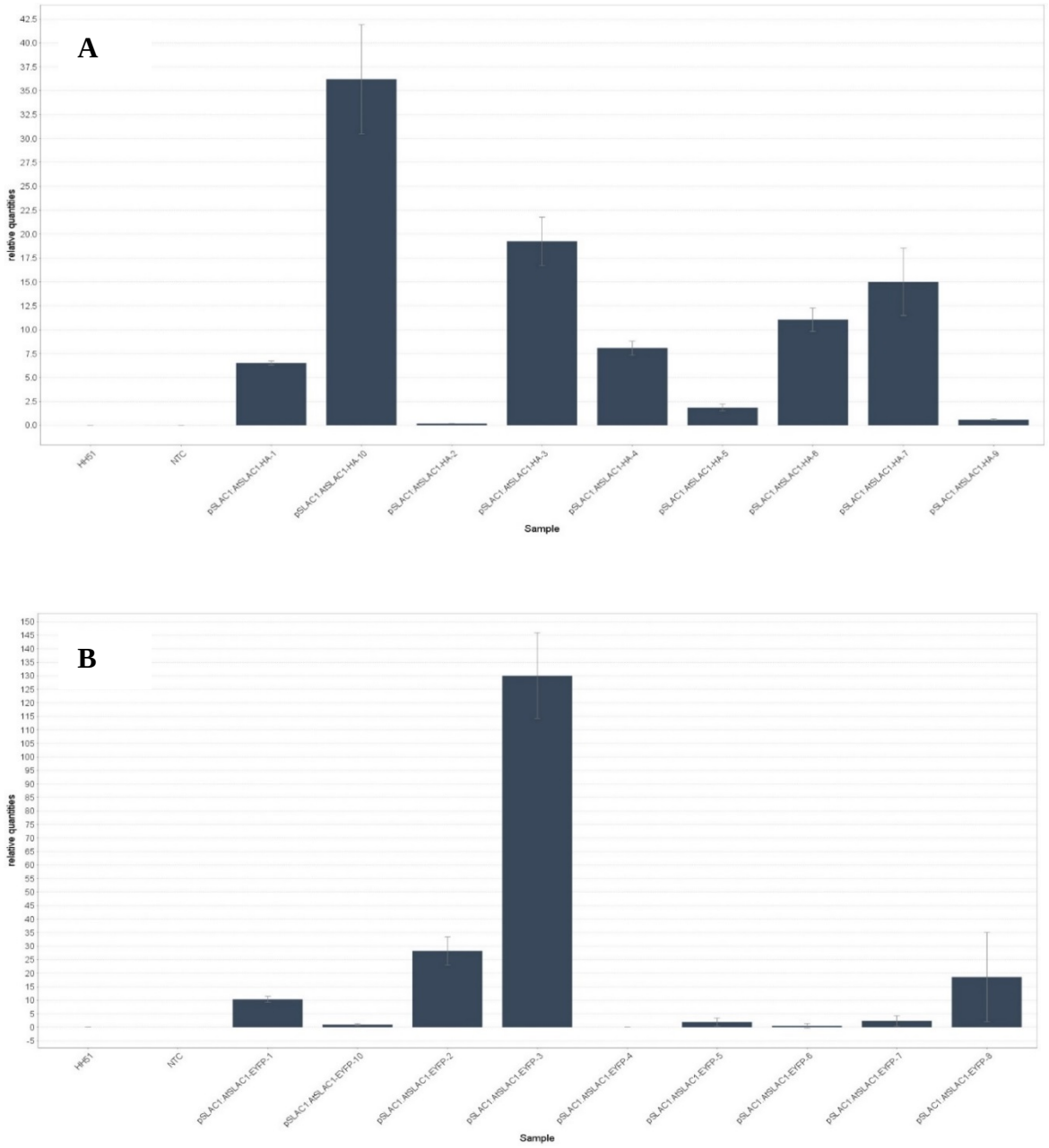

Figure 8: Expression of *SLAC1* in guard cells of transgenic lines containing *pSLAC1::AtSLAC1-HA* (A) and *pSLAC1::AtSLAC1-EYFP* (B) constructs. Transcript levels of SLAC1 was determined by qPCR. Three reference genes (TIP41-like, TUBULIN and ACTIN) were used for normalization. Data are the average of three biological replicates and for each replicate experiments were performed in triplicate. Bars represent ± SE for biological replicates. HH51 denotes control (untransformed) and NTC stands for non-template control.

6 DISCUSSION

In this study all the lines were first confirmed to be transgenic. Two primer pairs were used for this purpose. *ACTIN* is one of the abundantly expressed housekeeping gene and will be present in both wild type and transgenic plants. On the contrary, the other primer pair was used to amplify the part of the kanamycin resistance gene in transgenic plants to confirm that the plants are transgenic. The PCR analysis provided positive results for each independent lines of the four transgenic constructs, except for line number 6 and 11 for the construct *pGC1::AtSLAC1-HA* (Fig. 3). However, the missing band, corresponding to actin, for these two lines, suggested that there was problem with the PCR amplification as for those samples the DNA template was likely of low quality.

The expression pattern of *SLAC1* was observed using GUS reporter driven by two different promoters, *pSLAC1* and *pGC1*. By far, it was the first study where a guard cell-specific promoter was used in *Populus* (Sanna Ehonen, University of Helsinki, personal communication). The purpose of studying two different promoters was to find out, if these promoters are guard cell-specific in hybrid aspen, and more specifically, which one of these two promoters is more guard cell-specific. The promoters were chosen for this study based on previous results in Arabidopsis. *pSLAC1*, from *Arabidopsis thaliana*, which has been shown to be guard cells-specific (Vahisalu et al. 2008) and *pGC1*, from *P. trichocarpa*, were studied. The *P. trichocarpa* Potri.019G083900 gene is orthologue for the *Arabidopsis thaliana GC1* gene, which promoter has been shown to be guard cell-specific (Yang et al. 2008).

From the result of GUS assay it was found that *pSLAC1* is more guard cell-specific than *pGC1*. For *pGC1::GUS* lines the GUS activity was found in veins along with the guard cells, while GUS activity was largely confined to guard cells for *pSLAC1::GUS* lines. This study is in line with other well documented studies that *pSLAC1* is highly specific to guard cell (Negi et al. 2008, Vahisalu et al. 2008, Imai et al. 2015, Zheng et al. 2015).

The pattern of measured growth parameter, height and basal diameter, for each of the four genotype was similar. Significant differences in height increment were found. Differences in basal diameter was also significant, except for

pGC1::AtSLAC1-EYFP lines (Table 2). It was found that in all the genotypes most of the transgenic lines showed increase in height increment percentage and decrease in basal diameter compared to wild type. Water loss experiment was performed for *pSLAC1::AtSLAC1-HA and pSLAC1::AtSLAC1-EYFP* and expressed as percentage of fresh weight. Statistical analysis of water loss experiment suggested that there were no significant differences to wild type. However, after two hour time point, except three individual transgenic lines (*pSLAC1::AtSLAC1-HA-7* and *pSLAC1::AtSLAC1-EYFP-4, -6,* Table 1), all other lines tested, showed lower percentage of initial fresh weight than in wild type. This result is in contrast to other studies (Vahisalu et al. 2008, Imai et al. 2015), where they have shown that mutation in *SLAC1* resulted in higher percentage of initial fresh weight loss compared to wild type *Arabidopsis thaliana.*

SLAC1 gene is a central regulator of guard cell S-type anion channel and has been reported as crucial for stomatal closure in response to different stimuli, for example; CO_2, ABA, O_3, light and humidity (Vahisalu et al. 2008, Negi et al. 2008, Saji et al. 2008). In this study, *SLAC1* regulated stomatal conductance, in response to elevated CO_2 was examined only, the other factors contribute to stomatal closure such as relative humidity and light was kept constant. The stomatal conductance was measured and analyzed to observe how the different transgenic lines behave with the increase in CO_2 concentration. With the increase of CO_2 concentration, the transgenic lines expressing *AtSLAC1* gene showed a decline in stomatal conductance compared to wild type that is most likely devoid of functional *SLAC1*. Another study in *Arabidopsis thaliana* (Vahisalu et al. 2008) reported that *slac1* showed no response even after doubling the concentration of CO_2 from 400 ppm to 800 ppm while in wild type stomatal conductance was reduced rapidly.

Though the result of stomatal conductance showed a decline in transgenic lines compared to wild type, in response to elevated CO_2 concentration, the response was not consistent in all the biological replicates of a single independent transgenic line. The possible causes might be changes in environmental condition outside the greenhouse, position and age of leaves and leaf morphology. Changes in stomatal conductance measured on a cloudy day remained almost the same in response to elevated CO_2 concentration compared to measurements conducted

during sunny days. The time of the day might also influence the stomatal conductance, as in this study the measurements were conducted between 9.00 to 17.00 hours, whereas other studies reported (Mäenpää et al. 2011, Kusumi et al. 2012) stomatal conductance measurements mostly done between 10.00 to 16.00 hours. Another factor that might have been influenced the measurement is that during this experiment the plants were eight-weeks-old with very large leaves and the cuvette covered only small portion (8 cm^2) of the leaves, and thus the changing conditions outside the cuvette had a bigger effect on the behavior of the stomata than the changes that were introduced inside the cuvette.

SLAH3, a homologue to *SLAC1* has the similar protein structure as SLAC1 protein and is involved in ion homeostasis in guard cells. Studies have shown that *SLAH3* is also expressed in guard cells (Geiger et al. 2011, Zheng et al. 2015), although the expression in guard cells is weaker than expression in roots (REF). *SLAH3* has also been reported to be capable of mediating S-type anion channel in the presence of NO_3^- and is responsible for stomatal closure upon drought stress (Geiger et al. 2011). *SLAH3* is also present in *Populus*, and thus one speculation could be that the function of *Populus SLAC1* might be overtaken by *SLAH3*.

7 CONCLUSION

Stomata play crucial role in the acclimation and adaptations of plants to their environment. Stomatal closure in response to various biotic and abiotic stimuli is important. For example, rising atmospheric CO_2 causes reduction in stomatal apertures influencing leaf heat stress and water use efficiency during photo synthesis. *SLAC1*, which is regulating the S-type anion channel, has been identified as a central component of guard cell regulation.

The first aim of this study was to demonstrate that *SLAC1* promoter is more guard cell-specific than *GC1* promoter. The second aim was to screen transgenic hybrid aspen lines expressing Arabidopsis *SLAC1* gene, and its effect to rapid stomatal closure in response to elevated CO_2 concentration. It was confirmed that the lines were transgenic and that they responded to elevated CO_2 concentration. However, it was difficult to conclude which transgenic lines were the strongest ones to select, as all the biological replicates did not respond in the similar fashion. To select such transgenic lines, this study suggests to have a more detailed study in gas exchange experiment, using other stimuli such as ABA, O_3, light quality and humidity that influence *SLAC1*-dependent stomatal closure.

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APPENDIX 1: STOMATAL REGULATION IN RESPONSE TO ELEVATED CO_2 (HH51 vs *pSLAC1::AtSLAC1-HA*)

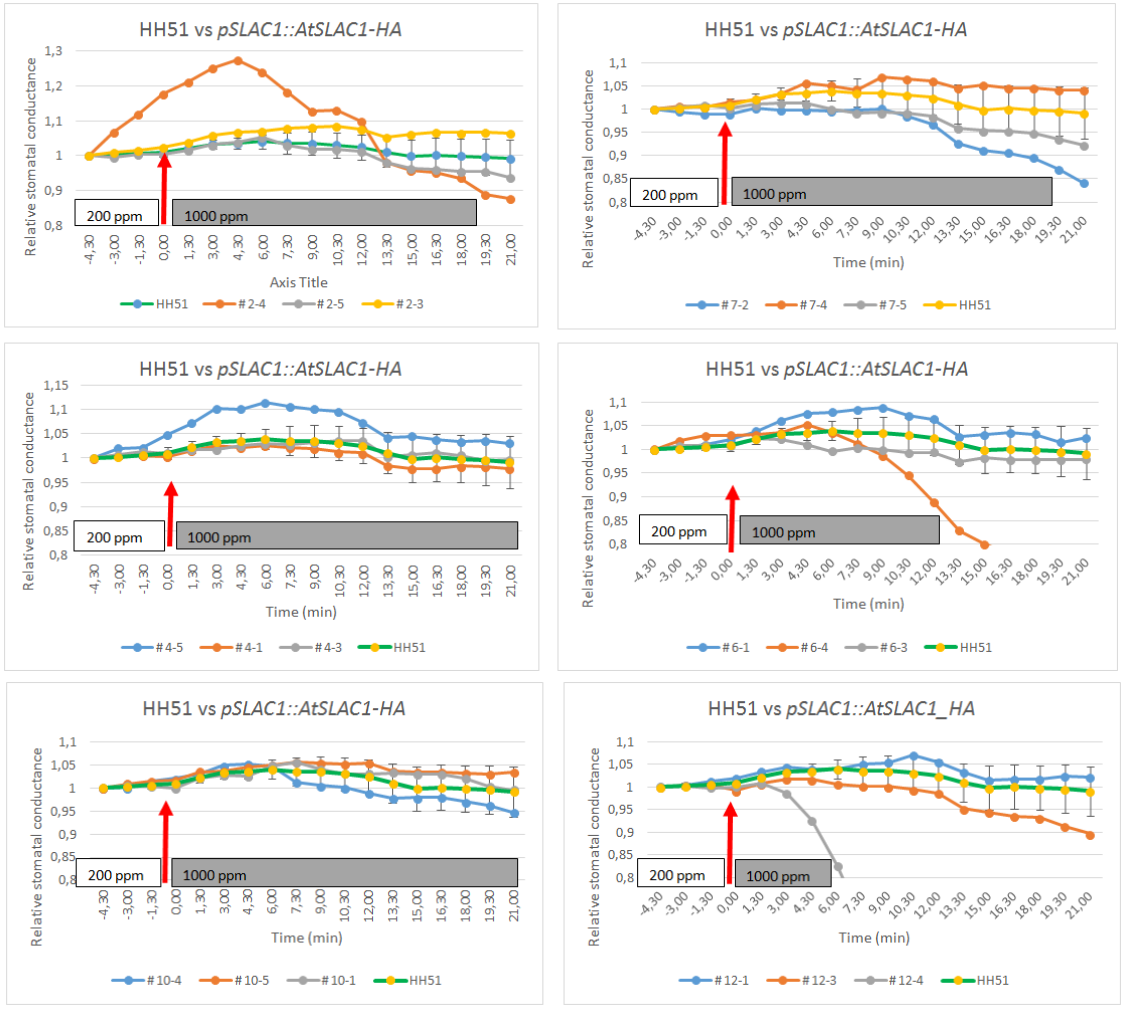

Figure 9. Relative stomatal conductance in response to changes in CO_2, in wild type (HH51) and transgenic lines, *pSLAC1::AtSLAC1-HA*. Data for wild type represent average of seven biological replicates ± SD.

APPENDIX 2: STOMATAL REGULATION IN RESPONSE TO ELEVATED CO_2 (HH51 vs *pSLAC1::AtSLAC1-EYFP*)

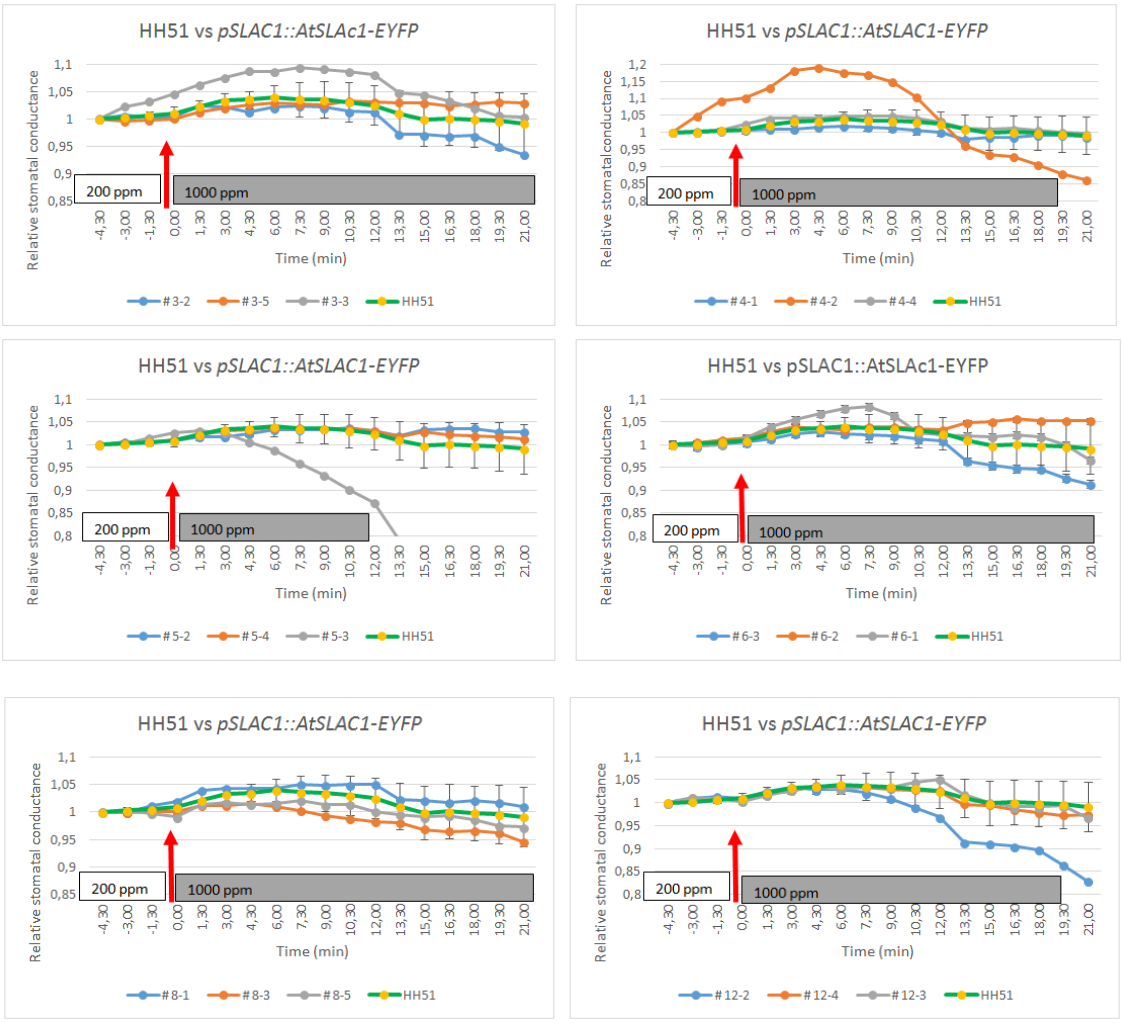

Figure 10. Relative stomatal conductance in response to changes in CO_2, in wild type (HH51) and transgenic lines, *pSLAC1::AtSLAC1-EYFP*. Data for wild type represent average of seven biological replicates ± SD.